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13. ABSTRACT (Maximum 200 Words)

The long-term objective of this project is to define the in vivo role of the receptor tyronsine kinase Ron in mammary gland biology. Virtually nothing is known regarding the function of Ron in the breast. However, two recent studies have shown the Ron is over-expressed and highly phosphorylated in a significant fraction of human and feline breast cancers. To define the in vivo significance of Ron, mice were generated with a targeted ablation of the tyrosine kinase domain of this receptor (TK-/-mice). To analyze the function of Ron in mammary development, mammary glands from wild type and TK-/- mice were collected at weekly intervals form 3 to 15 weeks of age. Based on whole mount and histological analyses, the TK-/- mammary glands display a markedly increased amount of epithelial branching that was most prominent at 6 weeks of age. To determine the in vivo impact of Ron in mammary gland tumors, wild type and TK-/- mice containing mammary expression of polyoma middle T antigen (pMT) were generated. Preliminary analyses indicate that Ron signaling augments pMT-induced mammary tumor formation and growth.

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Table of Contents

INTRODUCTION

In vitro studies have shown that Ron is a potent activator of cellular proliferation, migration, branching morphogenesis and invasion-characteristics that are critical for organogenesis and tumorigenesis. Further, alterations in Ron expression have been documented in a number of human tumors(1-8). However, the strongest link to Ron and human cancers may be in the breast. Recent studies have shown that Ron is expressed in the developing breast and Ron's expression pattern suggests that Ron may be influential in the development of this organ(9). In a study analyzing mammalian breast carcinomas, Ron was found to be expressed at abnormally high levels in a large fraction of primary human and feline tumors analyzed(2, 7, 10). Furthermore, in breast carcinomas grown in vitro, Ron activation resulted in proliferation, migration and invasion, suggesting a role for Ron in breast cancer progression and a metastatic phenotype(7). The purpose of this proposal is to determine the contribution of Ron signaling in normal mammary gland growth and development (Aim 1). This is being accomplished by contrasting breast development in wild-type mice compared to mice with a block in Ron signaling. Secondly, the impact of Ron signaling in the pathogenesis of oncogene-driven mammary gland tumors is being evaluated (Aim 2). This will be accomplished by monitoring tumor kinetics and downstream signaling cascades in a polyoma virus middle T antigen (pMT)-induced model of breast carcinogenesis.

BODY

Mammary Histology: In a histological analysis of the resting mammary glands of virgin mice containing a targeted ablation of Ron (referred to as the TK-/- mice), striking abnormalities were found (Figure 1). The mammary glands of TK-/- mice have an altered morphology consisting of ducts with an abnormal piling up of cells with large unevenly spaced nuclei, crowded lumens, and more extensive branching patterns. At present, we have collected several independent mammary glands at weekly intervals from 3 to 15 weeks from wild-type (TK+/+) and TK-/- mice. While we are in the process of analyzing these glands histologically and morphologically, similar alterations have been observed in TK-/- mammary glands from mice at 6 and 10 weeks of age with the most notable defects visible at 6 weeks of age (data not shown).

Consistent with our histological observations of the mammary gland, we have observed that the mammary fat pad of virgin TK-/- females appeared slightly thickened compared to controls. In order to analyze this in more detail, whole mount preparations were taken to examine ductal development. We observed an increase in duct density and branching in 6-week-old virgin TK-/- females compared to matched TK+/+ controls (Figure 2). As is apparent in Figure 2A, control TK+/+ female mammary glands display normal ductal morphogenesis, while the mammary glands from the TK-/- mice develop precociously. Gross inspection of the mammary glands from the TK-/- mice revealed an increased penetration of the ductal epithelium into the mesenchymal fat pad compared to control glands (Figure 2A versus 2B). In addition, the TK-/- epithelium has a denser network of branching ducts and has alveolar-like buds that resemble glands of early pregnant animals. Thus far, penetrance of this defect has been observed in 91% of all virgin TK-/- mammary glands analyzed (n=22).

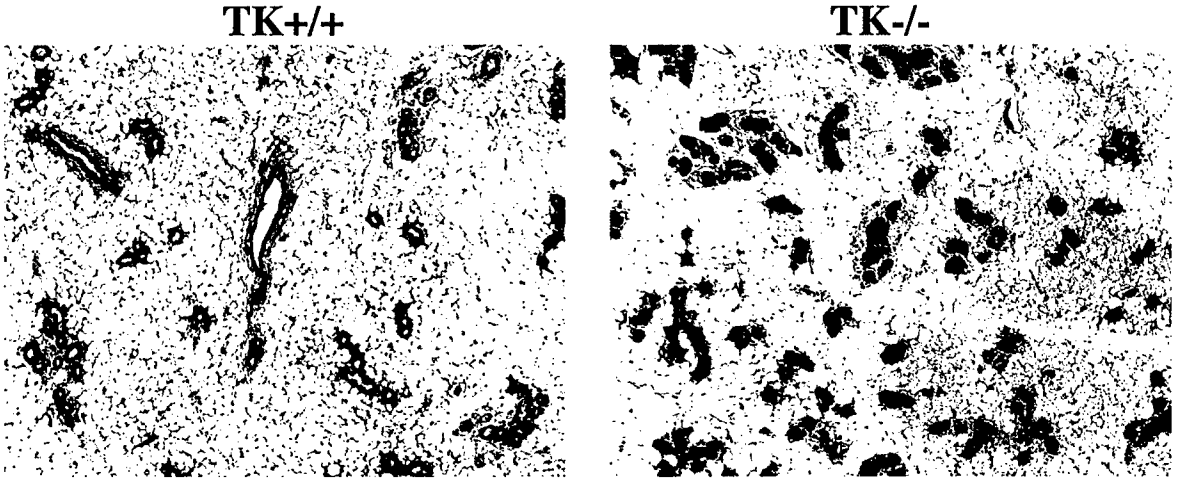

Figure 1. Abnormal mammary gland histology in the TK-/- mice. Histological sections of mammary glands taken from 6-week-old virgin Ron TK+/+ and Ron TK-/- mice. Note the increased number of branches in the TK-/- glands.

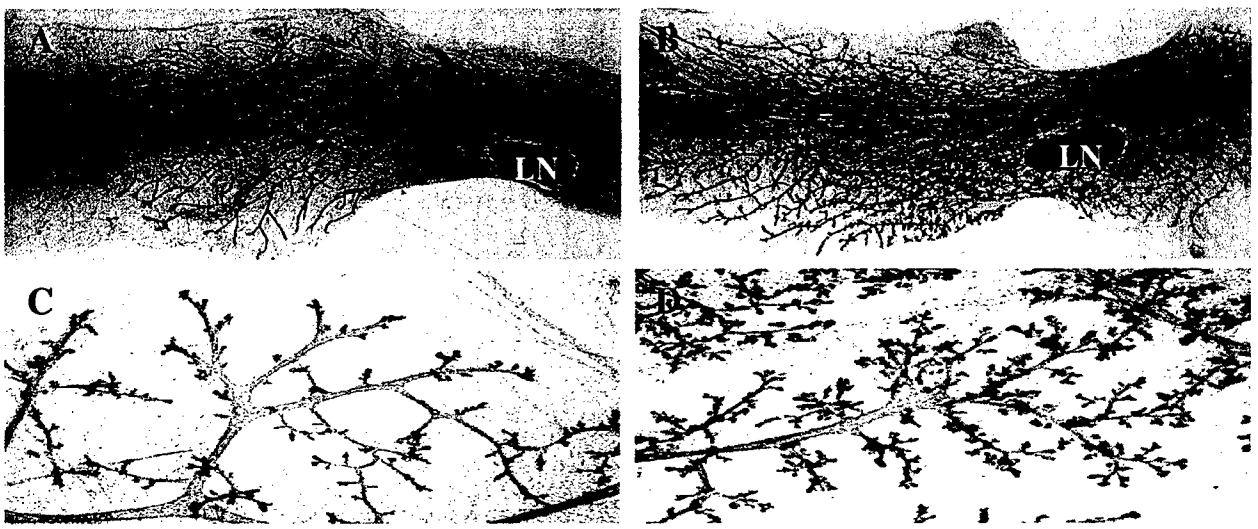

Figure 2. Abnormal mammary duct development in the Ron TK-/- mice. Whole mount analysis of the inguinal mammary fat pads of virgin 6 week-old female mice. Excessive ductal branching is seen in the Ron TK-/- mice (B and D) compared to age-matched control mice (A and C). LN, Lymph Node.

To examine whether loss of Ron signaling in the mammary glands of TK-/- mice has an effect on DNA synthesis, we performed in vivo bromodeoxyuridine (BrdU) incorporation assays (Figure 3). A significant increase in BrdU positive cells was found in the TK-/- mice compared to aged matched controls (6 weeks of age; $P < 0.02$). We counted over 600 mammary epithelial cells in corresponding areas of TK+/+ and TK-/- mammary glands and determined the percent of BrdU staining cells per total epithelial cell number. At 6 weeks, the TK+/+ mammary epithelial cells have an average of 4.35 % (+/- 1.25 SEM) BrdU positive staining cells, while the number of proliferating cells in the TK-/- mice is 3.2 fold higher (13.8% +/- 0.2). Although we see more extensive mammary development in the TK-/- mice, we have not observed any mammary tumors

or hyperplastic nodules. This data suggests that Ron may be important primarily during the postnatal development of the mammary gland. This is consistent with published reports showing that Ron is upregulated during the development of the mammary gland, but its expression is absent during pregnancy, lactation and involution(9). ***Thus, our preliminary analyses show that at 6 weeks of age, Ron TK-/- mice display precocious development of the mammary gland with the alveolar-like buds in virgin females resembling the glands of an early pregnant animal morphologically (see Figure 2D).***

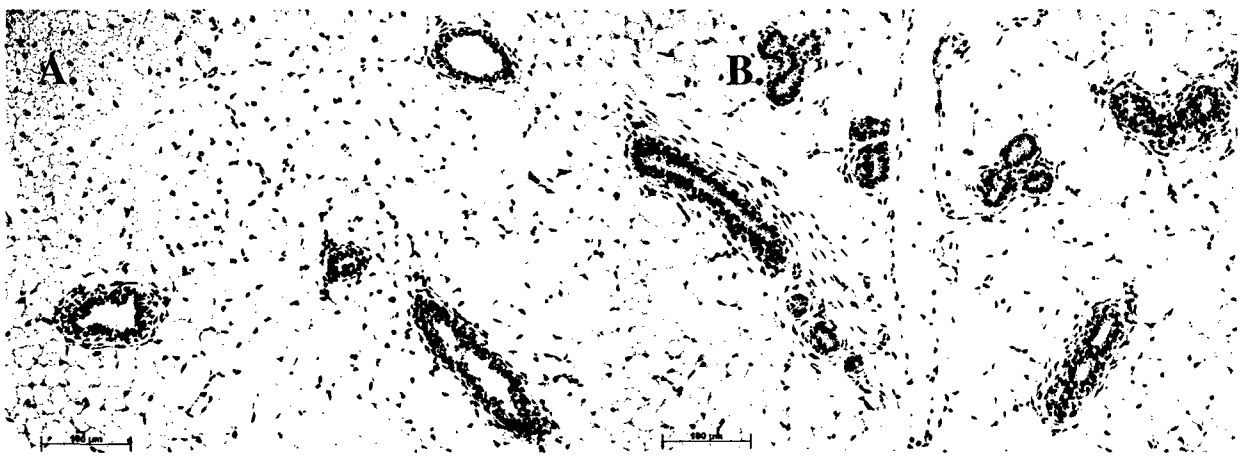

Figure 3. Increased mammary epithelial proliferation in Ron TK-/- mice. Six-week-old virgin TK+/+ (A) and TK-/- (B) mice were injected with BrdU intraperitoneally 2 hours before sacrifice. Paraffin sections of the inguinal (also referred to as the #4) mammary gland were immunostained with an anti-BrdU monoclonal antibody and detected utilizing a peroxidase system. Sections were counterstained with hematoxylin. Bar corresponds to 100μm. Note the significant increase in BrdU positive (black staining) cells in the TK-/- mammary epithelium.

Ron Expression in the Mammary Gland: While one published report has documented Ron expression in the mammary gland as a whole, the cells that express Ron in the mammary gland have not been analyzed. To determine the expression pattern of Ron in the mammary gland, RT-PCR and immunohistochemistry were performed. Our data demonstrate that Ron is expressed throughout the mammary gland, including in mammary epithelial cells (Figure 4). Primary mammary epithelial cells and fibroblasts were isolated from wild-type mammary glands according to established protocols. Figure 4A shows the morphology of the epithelial cells and fibroblasts isolated and grown in culture. We were able to isolate relatively pure populations of cells based on standard techniques with purities of about 95% or greater based on morphological characterization. RNA was isolated from both cell types and subjected to RT-PCR with Ron specific primers. The primers were designed to span intervening sequences so that PCR products from the Ron transcripts could be distinguished from contaminating DNA based on size. Figure 4B demonstrates Ron expression in both mammary epithelial and fibroblasts cells. No RNA specific product was observed in the absence of reverse transcriptase (-RT). In addition to PCR analysis, immunohistochemistry for Ron protein expression was performed on mammary gland sections of TK+/+ mice (Figure 4C). We detected Ron expression primarily in the mammary epithelium, however, faint Ron expression was observed throughout the entire gland. No specific staining was detected in the negative control sections.

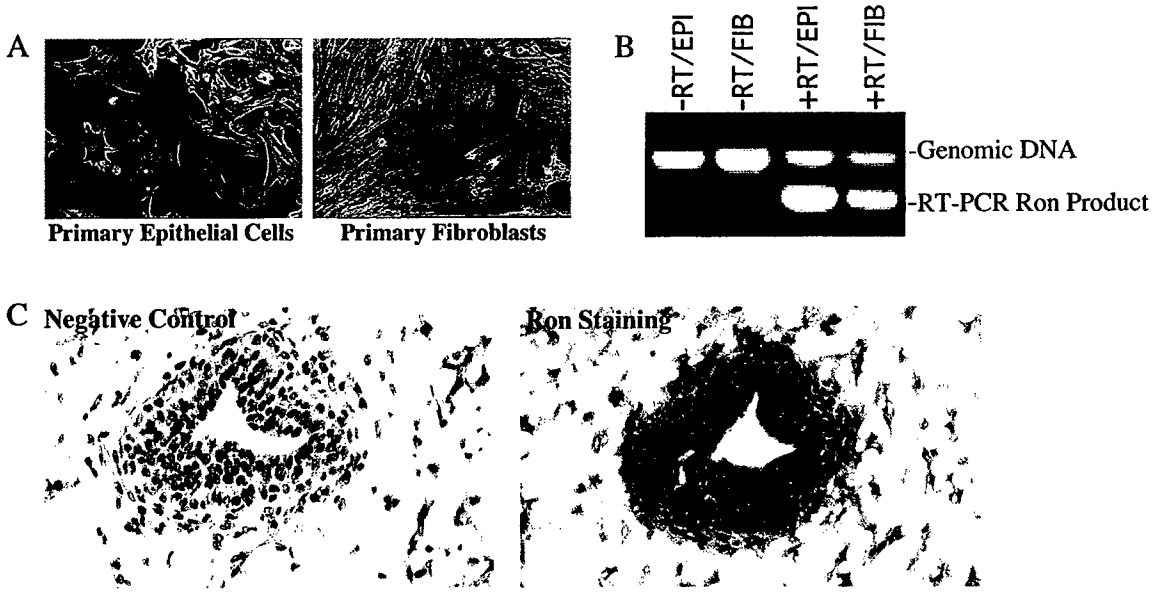

Figure 4. RON Expression and Localization. A, Isolation and culture of primary epithelial cells and fibroblasts from wild-type mammary glands. **B**, RT-PCR analysis. RNA was isolated from cultured primary mammary epithelial cells (EPI) and fibroblasts (FIB) from wild-type mice. RNA was subjected to RT-PCR in the presence (+) and absence (-) of reverse transcriptase (RT). The PCR products were analyzed by agarose gel electrophoresis. Sizes of the Ron mRNA product and genomic DNA are shown. **C**, Immunohistochemical staining for Ron in a TK+/+ 6 week old mammary gland. Note the Ron expression in the mammary epithelium (brown staining).

Decreased Tumor Formation in Mice with an Ablation of Ron Signaling: In order to test the hypothesis that Ron is involved in the pathogenesis of breast cancer in vivo, we have mated our TK-/- mice to mice containing mammary-specific expression of the Polyoma virus middle T antigen (pMT)(11). In this model, pMT expression is directed to the mammary epithelium by the mouse mammary tumor virus long terminal repeat (MMTV) promoter(11). PMT expression results in the widespread transformation of the mammary epithelium and the rapid production of multifocal mammary adenocarcinomas. Further, the majority of wild-type mice with tumors develop metastatic lung tumors. In a preliminary analysis with 8 TK-/-, pMT+/- and 8 TK+/+, pMT+/- female mice, we have observed differences in tumor onset and growth (Table 1). At approximately 85 days, TK+/+, pMT+/- mice had tumors in 70% of the mammary glands examined (56/80) while only 28% (22/80) mammary glands had tumors in the TK-/-, pMT+/- mice. Further, the TK-/-, pMT+/- mice have a reduced primary tumor burden (defined as the percent tumor weight per body weight). ***This data strongly suggests that Ron signaling deficiency results in a reduction of the primary tumor burden in this oncogene-driven model of mammary tumorigenesis.*** At present, more mice are being produced for further analysis.

Table 1: Tumor Frequencies and Growth Characteristics.

Genotype	Tumor Frequency	Days to Palpable Tumor Formation	Primary Tumor Burden	#Glands with Tumors/Total Glands
TK+/+, pMT+/-	8/8	46 (+/-2.9)	12.6% (+/-2.4)	56/80
TK-/-, pMT+/-	5/8	54 (+/-4.8)	5.9% (+/-1.5)	22/80

At first this tumor data may not seem congruent with the increases in proliferation we observed in the TK-/- mice (without pMT expression, Figure 3). However, the increased development of the mammary glands in the TK-/- mice is consistent with the premise that loss of Ron signaling directs the mammary epithelium toward a more mature or differentiated phenotype. The mature epithelium of the TK-/- mice may then be less sensitive to transformation by pMT expression compared to wild-type mice. This premise is consistent with results obtained analyzing Met in mammary cell lines(12). A reduction in Met signaling through the use of dominant negatives lacking the tyrosine kinase domain of Met, in DA3 mammary cells resulted in an increase in cell proliferation and induced in vitro and in vivo tubulogenesis. Tubule formation is a major characteristic of differentiated mammary cells. Thus, the reduction in Met enabled DA3 cells to form branching tubular structures. In addition, dominant negative forms of Met were found to reduce the in vitro motility and invasiveness, as well as in vivo tumorigenic and metastatic potential of transfected DA3 cells.

***Ron Expression in pMT-induced Tumors*:** To demonstrate the expression of Ron in mammary tumor tissue of the pMT-expressing mice, RT-PCR and immunohistochemical analyses were performed. RNA was isolated from mammary tumor tissue from a TK+/+, pMT+/- mouse and subjected to RT-PCR with Ron specific primers (as in Figure 4B). Figure 5A demonstrates Ron expression in the pMT-induced mammary tumors (+RT lane). No Ron expression was detected in control reactions without reverse transcriptase (-RT). We have also performed Northern analyses and showed there was no difference in pMT expression between the TK+/+ and TK-/- mice indicating that differences in tumor size are not a result of changes in pMT expression between genotypes (data not shown). In agreement with the RT-PCR analyses, immunohistochemistry was performed on paraffin embedded sections of tumors from wild-type mice expressing the pMT transgene (Figure 5B). Ron protein expression was observed in the tumor tissue of these mice.

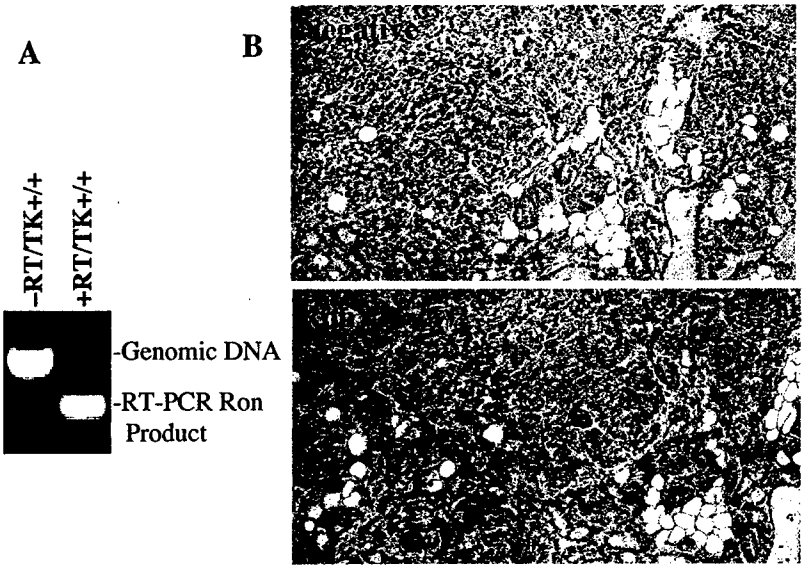

Figure 5. Ron Expression in pMT-Induced Mammary Tumors. **A**, RT-PCR was utilized to detect Ron expression in a TK+/+ pMT+/- tumor sample in the presence (+) or absence (-) of reverse transcriptase (RT). The sizes of the expected Ron mRNA and genomic DNA products are shown. **B**, Immunohistochemistry with an isotype control (top) and anti-Ron antibody (bottom) on sections of a mammary tumor from a TK+/+ pMT+/- mouse. The red bar represents 50um.

Summary: In summary, our data show that Ron is expressed in wild-type mammary epithelial cells and is expressed in pMT induced mammary tumors. We have also demonstrated that a lack of Ron signaling in vivo results in alterations of mammary gland development (Figures 1-3). Finally, our preliminary data suggests that Ron signaling augments the progression of oncogene induced mammary tumor formation (Table 1).

KEY RESEARCH ACCOMPLISHMENTS

- The Ron receptor is expressed through out the mammary gland, including the mammary epithelium.
- Ablation of Ron signaling results in defects in normal mammary gland development.
- Ron expression is upregulated in the mammary epithelium during polyoma middle T antigen (pMT)-induced mammary tumorigenesis.
- Ron signaling augments pMT-induced breast cancer initiation and growth.

REPORTABLE OUTCOMES

One reportable outcome resulting from the research supported by the US Army Medical Research and Material Command is a variety of mouse models. Specifically, we have generated mice containing mouse mammary tumor virus driven expression of the polyoma middle T antigen in combination with mice containing a specific loss of the tyrosine kinase domain of Ron. These pMT+/-, TK-/- mice may prove to be a valuable animal model system to define the role of Ron signaling during breast cancer progression.

In addition to research materials, this Career Development Award had led to an additional grant application from the P.I. The P.I. submitted a revised R01 grant application to the National Institutes of Health/National Cancer Institute in March of 2003. Additional supplementary material was supplied May 20, 2003 that supported the hypotheses and specific aims of the original application. The status of this grant is still pending, however, the application received a percentile of 4.8% and is likely to be funded.

Finally, this Career Development Award has also aided the P.I. in obtaining her current position as an Associate Professor in the Department of Surgery at the University of Cincinnati College of Medicine. When the P.I. applied for her Career Development Award, she was an Assistant Professor of Pediatrics at the Cincinnati Children's Hospital Research Foundation. With help of this award, the P.I. was able to compete for and obtain this promotion to her current position.

CONCLUSIONS

Our data indicate that Ron receptor signaling may be a new and important therapeutic target in our fight against breast cancer. We have demonstrated that Ron is expressed in the normal mammary epithelium and its expression is increased in a model of oncogene-induced breast cancer. This expression pattern is observed in human and feline cancers. Further, we have shown that Ron signaling participates in normal mouse mammary gland development, regulating branching morphogenesis. This data is the first to directly demonstrate a novel role for this receptor in the mammary gland. As we learn more about Ron regulated signaling events in the breast, we will be in a better position to understand how this organ develops and the processes involved in mammary tumorigenesis and metastasis.

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APPENDICIES

No appendices are included in this report.